

LEARNED PREDATOR RECOGNITION IN A FRESHWATER SNAIL, *POMACEA CANALICULATA*

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ABSTRACT

The involvement of associative learning in predator recognition has not been clear in aquatic invertebrates, including molluscs, due to confounding effects of sensitization. The freshwater apple snail, *Pomacea canaliculata*, displays an alarm response (crawling above the water-line) when exposed to crushed conspecifics or some predators. We conducted two series of experiments to investigate whether the apple snail learns to avoid predators. In the first experiment, hatchlings were conditioned simultaneously to crushed conspecifics and either a live carp, *Cyprinus carpio*, or a turtle, *Chinemys reevesii*, and subsequently exposed to the same predator without crushed conspecifics. Irrespective of the predator species used, the alarm response was significantly higher in conditioned snails than in unconditioned snails. Thus, the snail is able to avoid predators by learning, in a broad sense. In the second experiment, designed to distinguish associative learning from sensitization, we conditioned hatchlings to crushed conspecifics and either a carp or a turtle. The hatchlings were subsequently exposed to one or other of the predators. Hatchlings that were conditioned to a predator displayed significantly higher alarm response when later exposed to the same predator than another predator, suggesting that the snail can recognize predators by associative learning.

Key words: alarm response, antipredator defense, apple snail, associative learning, Caenogastropoda.

INTRODUCTION

Prey often alter their behavior in the presence of predators to improve their chances of survival (Kats & Dill, 1998). Such predator-induced alarm responses have been well studied in prey species as they may affect life history strategies, such as changes in growth and reproductive patterns (Crowl & Covich, 1990). Alarm responses may also affect local community structures via trait-mediated indirect interactions (Turner et al., 2000; Trussell et al., 2002).

To survive predation with minimum cost, prey species should exhibit flexibility in their alarm responses, responding to both innate and learned predator cues (Ferrari et al., 2005, 2008). Learning of predator recognition, in the broad sense (i.e., change in behavior in response to experience; Decolmyn, 1998), has been reported in several aquatic animals, including polyclads (Wisenden & Millard, 2001), gastropods (Rochette, 1998; Dalesman et al., 2006; Turner et al., 2006), crustaceans (Hazlett,

2007), insects (Wisenden et al., 1997; Ferrari et al., 2008), fish (Ferrari et al., 2005, 2006, 2007), and amphibians (Ferrari & Chivers, 2008).

However, such behavioral changes may not be caused solely by associative learning between an alarm cue and a specific predator cue. For example, to study predator recognition learning in invertebrates the prey are typically exposed simultaneously to an innate alarm cue, such as injured conspecifics, and a predator cue, such as odor. The response of the prey to the predator cue alone is then tested. This design does not eliminate the possibility of prey sensitization caused by the alarm cue. Sensitization is a simple form of learning in which animals become sensitive to almost any stimulus after they experience a strong noxious stimulus, lasting for several minutes (Decolmyn, 1998) to more than a week (Walters, 1987). In some studies on predator recognition learning, cues that are not used for conditioning are also tested (Wisenden et al., 1997). However, the possibility remains that only the predator cue used in conditioning is strong enough to elicit

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an alarm response after being sensitized. In yet another type of study, the quantity of the innate alarm cue is varied during conditioning to the predator cue. The prey is subsequently shown to respond according to the quantity of the alarm cues used during conditioning (threat-sensitive learning; Ferrari et al., 2008). However, this might indicate that the level of sensitization depends on the quantity of alarm cues. These studies are not intended to distinguish between associative learning and sensitization. However, this distinction is important to support the implications of these studies that prey actually learn to recognize a specific predator rather than that they simply respond to any stimulus above a threshold after being sensitized by the alarm cue. Sensitization in defensive behaviors, such as gill or siphon withdrawal in the marine gastropod *Aplysia*, is a classical example of learning (Decolmyn, 1998) and observed in invertebrates such as gastropods (Walters, 1987; Decolmyn, 1998), crustaceans (Pijanowska et al., 2006), or insects (Walters et al., 2001). Therefore, sensitization is a likely confounding factor in studying predator recognition learning.

Although there are reports in fish (Chivers & Smith, 1994; Ferrari et al., 2005) and amphibians (Ferrari & Chivers, 2008), no study has clearly demonstrated the occurrence of associative learning, instead of sensitization, in predator avoidance in aquatic invertebrates, including molluscs. Associative learning in other behavioral responses has been well documented in molluscs (Alkon, 1999; Hochner et al., 2006), and hence they are likely to use associative learning in predator recognition as well. One method of distinguishing associative learning from sensitization is the use of double conditioning (Chivers & Smith, 1994). This involves the conditioning of two groups of prey individuals to an alarm cue from conspecifics and either of two different predators. The response of the prey to both predators is subsequently observed for each group. In this experimental design, if associative learning is involved, the prey should display a higher intensity of alarm response towards the predator they are conditioned to than towards the other predator. However, if sensitization to the alarm cue occurs, this will raise the intensity of the alarm response in all the treatment groups in a similar way, since all are treated with the same alarm cue.

We evaluated the mechanism of predator recognition in the freshwater apple snail,

Pomacea canaliculata (Caenogastropoda: Ampullariidae), using the double predator conditioning. The apple snail is highly invasive and is now distributed in the Americas and Asia (Joshi & Sebastian, 2006). Hatchlings crawl out of the water when they are exposed to an alarm cue from crushed conspecifics (Ichinose, 2002; Ichinose et al., 2003). However, they also respond to the odours of local predators, such as the climbing perch, *Anabas testudineus*, and the wetland crab, *Esanthelphusa nimoafi*, in non-native areas (Carlsson et al., 2004). This suggests that the apple snail can learn to avoid predators. The ability of learning may be especially important in invasive species that face many novel predators (Hazlett, 2007). In this study, two predators were used, the common carp, *Cyprinus carpio*, and the freshwater turtle *Chinemys reevesii*, both of which are efficient predators of the snail in Japan (Yusa et al., 2006; Yoshie & Yusa, 2008).

MATERIALS AND METHODS

Snails

We collected approximately 100 adult apple snails from paddy fields and canals in Kashiwagi-town, Nara, western Japan (34°40'N, 135°47'E) between July and October 2007. We reared the adults in an aquarium (60 x 30 x 35 cm, *l* x *w* x *h*) containing 60 liters of aerated water at 25–30°C under a natural photoperiod. The snails were fed almost daily with fish food pellets (Hikari Mini Pet, Kyorin, Himeji, Japan). We monitored the aquarium for evidence of egg laying. Each egg mass was transferred to a Petri dish and allowed to hatch at 25°C and under a 14L:10D photoperiod. The hatchlings from each egg mass were transferred to a plastic cylindrical mesh basket (9 cm diameter and 12 cm height) that allowed water exchange but did not allow the snails to escape. We placed the baskets in aquaria (33 x 19 x 21 cm) containing 3 liters of aerated, purified water (chloride, lead, and trihalomethane free). We fed the hatchlings daily with boiled flour balls (~5 mm in diameter; used in Experiment 1 with carp as the predator) or fish food pellets (used in Experiment 1 with turtles and Experiment 2). A preliminary experiment revealed that the type of diet did not affect the number of snails displaying an alarm response to the turtle, either with or without crushed conspecifics ($N = 11$, $F_{1,6} = 0.01\text{--}0.46$; $P = 0.52\text{--}0.92$; ANOVA

with egg mass as a random factor). Juveniles 3–8 days after hatching were used, with no previous experience with predators or crushed conspecifics. Nineteen egg masses were used in the experiments (5–8 for each experimental series), and in most cases, snails from each egg mass were used in all treatment groups.

Predators

We used juvenile carp (1.3–4.8 g) and turtles (5.2–17.4 g). They were obtained from pet shops, except for a carp individual captured from a river in Nara, where the apple snail was not present. Approximately 20 individuals of each species were held in an aquarium (35 x 20 x 25 cm) and acclimated for at least 1 day under the experimental conditions (25°C; 14L:10D photoperiod). The carp were fed tropical fish flakes (Pack de Flakes, Gex, Osaka). The turtles were fed the same diet as the apple snail. Food was withheld for at least 1 day prior to the experiments to avoid the confounding effect of food odour. We used the same predator individual for each replicate throughout the experiment. We attempted to minimize the repeated use of a single individual in each experimental series. However, in some cases it was difficult to identify individual predators over a long period. These animals were reared following the instruction of the Animal Experiment Committee of Nara Women's University.

General Preparation

All experiments were conducted from 08:00–13:00 between August 2007 and April 2008, and each experimental series lasted less than 2 months. One day before the experiment, 10 apple snail hatchlings, from a single egg mass, were taken randomly from the rearing basket and placed in another mesh basket that was held in an aquarium (35 x 20 x 25 cm) containing 5 liters of purified water (5.6 cm deep). The water was aerated and maintained at $25 \pm 1^\circ\text{C}$ throughout the experiment. The snails were supplied with an adequate amount of food and held under a 14L:10D photoperiod. Thirty minutes before the experiment, we removed the food and gently placed all snails that were above the waterline back into the water.

Experiment 1: Conditioning to a Single Predator

There were two experimental series, one using the carp as a predator throughout the

experiment and a second using the turtles. In each series, the experiment was divided into two phases (conditioning and recognition) and the experiment was conducted over a period of 5 days (days 1–5). The hatchlings in each experimental series were divided into two treatment groups (test and control). On day 1, we recorded the number of snails whose bodies were completely out of the water prior to the experiment. We then introduced a single predator (either a carp or a turtle depending on the experimental series) gently into the aquarium, outside the basket. The predator was rinsed with purified water prior to being placed in the aquarium to minimize the possible effect of the rearing water odour (recognition trial). We recorded the number of snails above the waterline 1 h after the experiment began. The duration of the treatment was chosen because the alarm response of the apple snail is highest between 15 min and 1 h (Ichinose et al., 2003), and we wanted to treat the snails with the predator or alarm cues for a long duration. The predator was then removed, the water was changed, and the snails were fed. On day 2 (conditioning trial), we recorded the position of the snails, and then introduced the same predator individual into the aquarium. In addition, we also placed a perforated plastic bag (9.5 x 7.5 cm) containing five crushed hatchlings from the same egg mass as those tested into the aquarium (test treatment). The control treatment consisted of a single predator and an empty bag, without crushed hatchlings. The number of snails above the waterline was recorded 1 h after the introduction of the predator and the bag. At the conclusion of the experiment, we removed the predator and the bag, changed the water, and fed the snails. On days 3 and 5, we repeated the recognition trial (predator only). Dead snails (less than 1% of individuals tested) were removed during the experiment. The experiment was replicated 11 times using the carp, and 14 and 13 times for the test and control treatments, respectively, using the turtle. (One control replicate of the experiment using the turtle was lost because extract from crushed conspecifics was entered by mistake.)

Experiment 2: Double Predator Conditioning

This experiment was divided into two phases (conditioning and recognition trials) over a period of 3 days (days 1–3). On day 1 we conditioned 10 snails in each aquarium to five

crushed conspecifics (also from the same egg mass) and a single predator (either a carp or a turtle). After 1 h we removed the predator and the crushed conspecifics, changed the water, and fed the snails. On day 3 we tested the response of the hatchlings to either a carp or a turtle (recognition trial). Thus, each replicate ($N = 10$ snails) was assigned to one of the following four treatment groups based on the predator used during conditioning/recognition trials: carp/carp (CC), turtle/carp (TC), carp/turtle (CT), and turtle/turtle (TT). There were 8 replicates for the CC, TC, and CT treatments and 7 replicates for the TT treatment (due to the death of a turtle in the TT treatment). We did not use control treatments (no treatment on day 1 and then exposed to each predator on day 3, as in Experiment 1) because our objective was to distinguish associative learning from other components by comparing the above four treatments.

Statistical Tests and Predictions

The number of snails displaying an alarm response was defined as the number of individuals above the water 1 h after the introduction of the predator minus the number prior to the introduction. The intensity of the alarm response was measured by the proportion, that is, the number of snails displaying the alarm response divided by the total number in each replicate ($N = 10$ in most cases). The data were $\log_{10}$ transformed when they violated the assumptions of normality and homoscedasticity, checked by Shapiro-Wilk test and Bartlett test, respectively. All statistical tests were two-tailed, and conducted using JMP IN 5.0 (Sall et al., 2004).

We compared the proportion of individuals displaying an alarm response in Experiment 1 between the test and control treatments. We used one-way ANOVA with the treatment (test or control) as the independent factor, with the egg mass identity added as a random factor (Grafen & Hails, 2002) using the REML option in JMP. By incorporating this random factor, we could statistically control the difference in the alarm response among egg masses or through time (as egg masses were laid over maximum 2 months). We focused the statistical test on the response of snails at the first recognition trial after conditioning (on day 3): if predator avoidance occurs as a result of learning, in the broad sense, a significant difference between the treatments should have been observed on day 3. However, we also studied the difference on

the other days to check the requirements of the experiment (no difference on day 1 and a significant difference on day 2), and the duration of learned predator avoidance as descriptive purposes (on day 5). By focusing the critical test on day 3, we avoided the problems of multiple comparisons and the interdependence of the data over experimental days.

In Experiment 2, we compared the intensity of the alarm response using two-way ANOVA, with the identity of the egg mass added as a random factor. In the recognition trial on day 3, we tested the effect of predator species used (carp or turtle) (termed "predator used"), whether or not the same predator was used in the conditioning and recognition trials ("consistency of the predator"), and the interaction term between them. If associative learning is involved, then "consistency of the predator" should have a significant effect. The "predator used" term reflects the difference in the intensity of immediate response of the snails to each predator. The interaction term corresponds to the effect of predator used in the conditioning trial on day 1, including sensitization to the predator. Note that these effects are not mutually exclusive.

To check the requirements of the experiment, we also tested the effects of "predator used" (on day 1), "consistency of the predator", and their interaction term in the conditioning trial on day 1. The "predator used" tests the immediate response to the predator for the first time (likely to be an innate response), and "consistency of the predator" and the interaction term test if there are pre-existing differences in the alarm response among treatment groups.

RESULTS

Experiment 1: Conditioning to a Single Predator

In the experiments using the carp, there was no difference in the proportion of snails displaying an alarm response between the test and control treatments in the recognition trial on day 1 ($F_{1,15} = 0.13$, $P = 0.73$ after $\log_{10}$ transformation; Fig. 1). In the conditioning trial on day 2, the proportion of snails displaying an alarm response in the test treatment (carp and crushed conspecifics) was significantly higher than in the control treatment (carp only) ($F_{1,15} = 71.52$, $P < 0.001$). Similarly, on day 3, the alarm response in the test treatment group was significantly greater than in the control

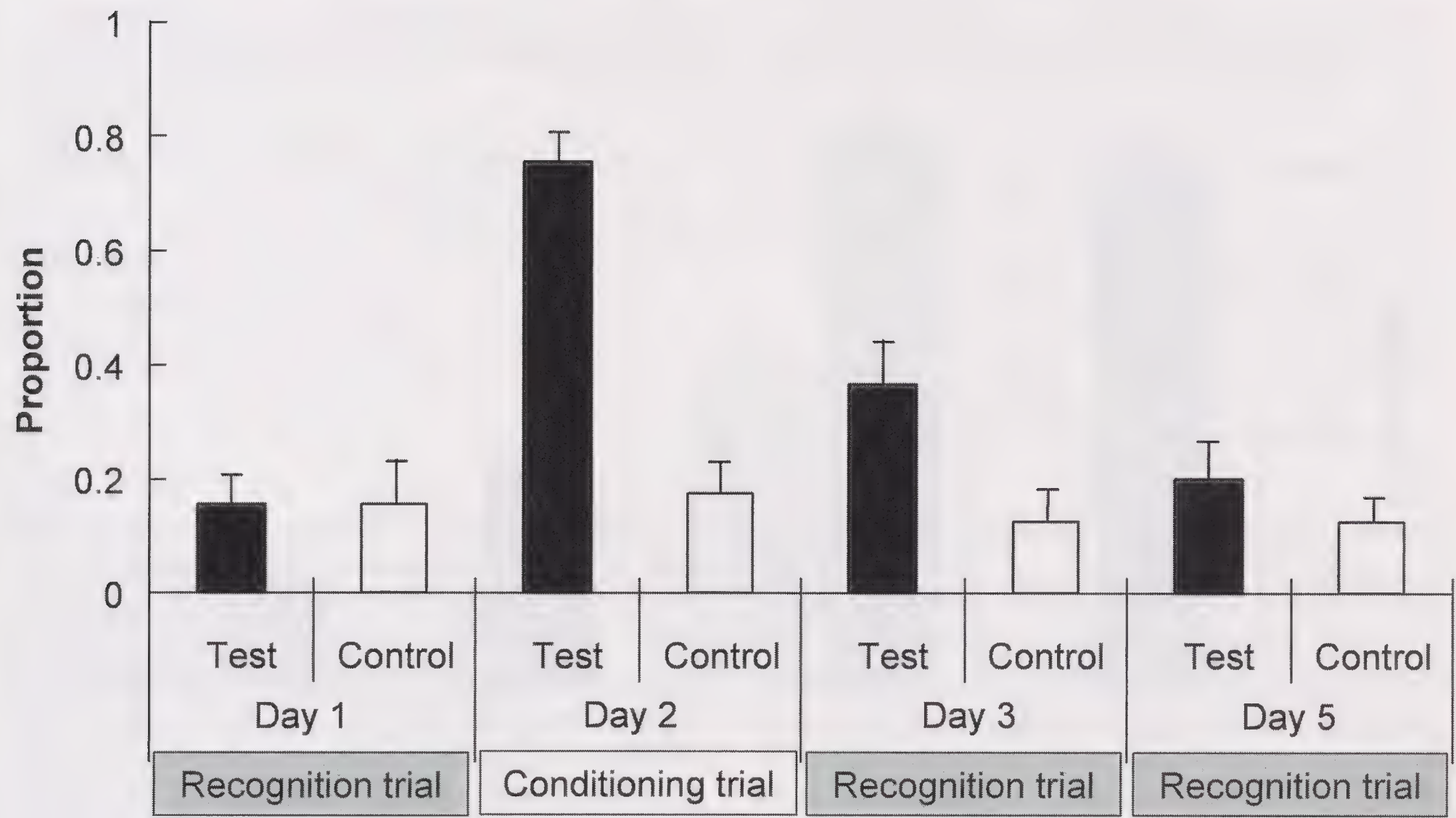


FIG. 1. Proportion (mean + SE) of snails displaying an alarm response in Experiment 1 (carp). Test snails were treated with crushed conspecifics and a live carp on day 2 and with the carp alone on other days, whereas control snails were treated with a live carp alone throughout. N = 11 for both test and control treatments.

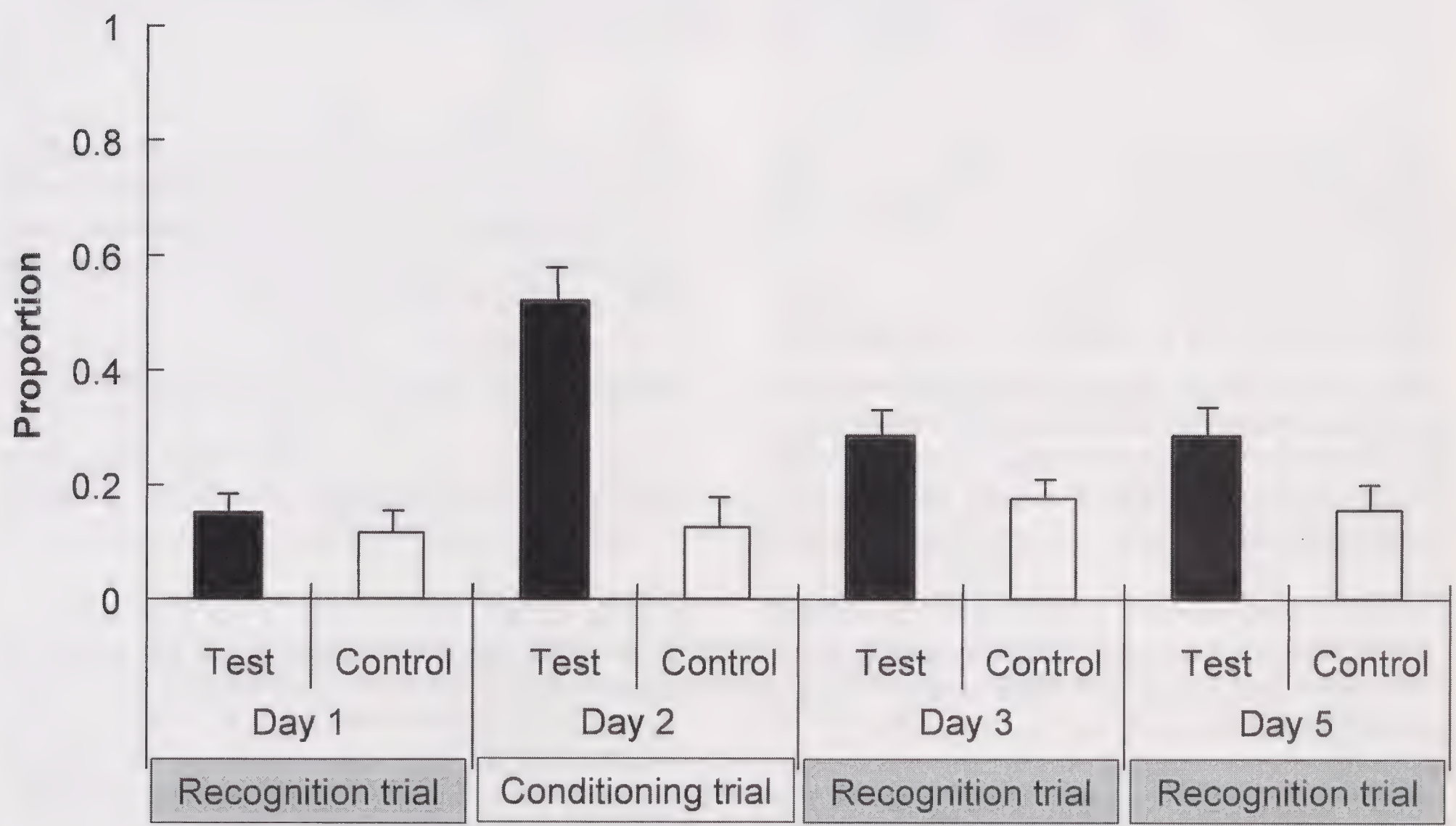


FIG. 2. Proportion (mean + SE) of snails displaying an alarm response in Experiment 1 (turtle). Test snails were treated with crushed conspecifics and a live turtle on day 2 and with the turtle alone on other days, whereas control snails were treated with a live turtle alone throughout. N = 14 for test treatment and N = 13 for control treatment.

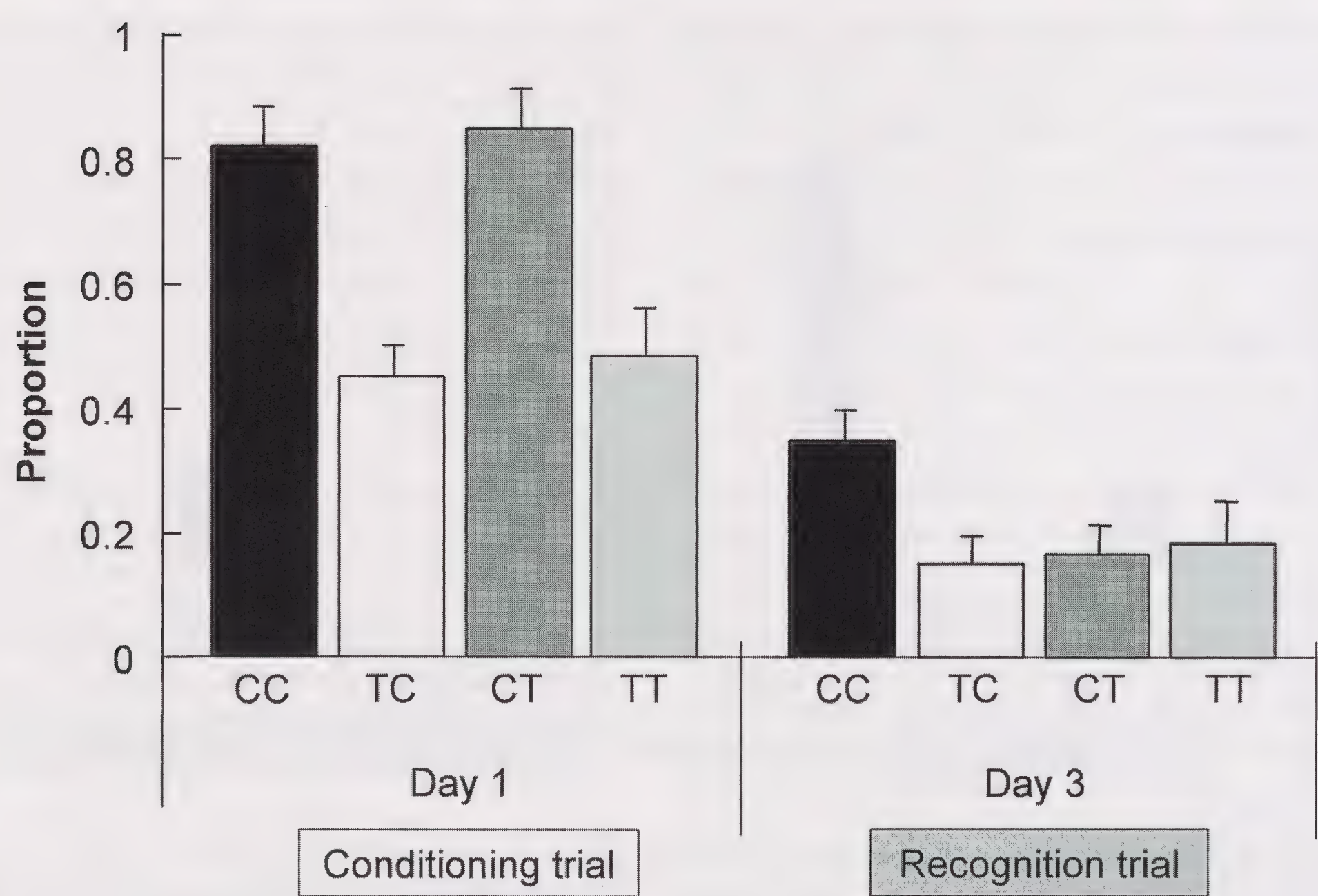


FIG. 3. Proportion (mean + SE) of snails displaying an alarm response in Experiment 2. The first letter in each treatment group indicates the predator used in conditioning with crushed conspecifics on day 1 (carp or turtle), and the second letter indicates the predator used in the recognition trial on day 3. N = 7 for TT treatment and N = 8 for other treatments.

treatment ($F_{1,15} = 8.62$, $P = 0.010$). There was no significant difference between the test and control groups on day 5 ($F_{1,15} = 1.26$, $P = 0.28$ after $\log_{10}$ transformation).

In the experiments using the turtle, the pattern was similar to the experiments using the carp. The proportion of snails displaying an alarm response was not different between the test and control treatments in the recognition trial on day 1 ($F_{1,20} = 2.06$, $P = 0.17$; Fig. 2). The proportion was higher in the test treatment than

in the control during the conditioning trial on day 2 ($F_{1,20} = 27.45$, $P < 0.001$). The difference was also significant during the recognition trial on day 3 ($F_{1,20} = 4.46$, $P = 0.047$), but not on day 5 ($F_{1,20} = 3.71$, $P = 0.066$).

Experiment 2: Double Predator Conditioning

The intensity of the alarm response was high during conditioning to a carp and crushed conspecifics on day 1 (Fig. 3). Between 83 and

TABLE 1. Results of an ANOVA testing the effects of “predator used”, “consistency of the predator” and their interaction term on alarm responses of snails during the conditioning trial (day 1) in Experiment 2.

Source	df	MS	F	P
Predator used on day 1	1	1.0400	40.00	< 0.001
Consistency of the predator	1	0.0004	0.017	0.90
Predator used x Consistency	1	0.0082	0.315	0.58
Error	23	0.0260		

TABLE 2. Results of an ANOVA testing the effects of “predator used”, “consistency of the predator” and their interaction term on alarm responses of snails during the recognition trial (day 3) in Experiment 2.

Source	df	MS	F	P
Predator used on day 3	1	0.0436	2.28	0.140
Consistency of the predator	1	0.0972	5.07	0.034
Predator used x Consistency	1	0.0594	3.10	0.092
Error	23	0.0192		

85% of the snails crawled out of the water in the CC and CT treatments, respectively. The intensity of the alarm response was lower when the snails were conditioned to a turtle and crushed conspecifics (45 and 49% for the TC and the TT treatments, respectively). The results of an ANOVA (Table 1) suggest that the identity of the predator used had a significant effect on the intensity of alarm response. Neither the “consistency of the predator” nor the interaction term had a significant effect (Table 1), suggesting that there was no pre-existing bias prior to the recognition trial.

The snails that had been conditioned to a carp on day 1 and subsequently exposed to a carp on day 3 (the CC treatment) had the highest alarm response (Fig. 3). The next highest was in the TT treatment, followed by the CT and TC treatments, although the difference among these three treatments was small. As a result of an ANOVA, “consistency of the predator” (i.e. whether or not the same predator was used throughout) had a significant effect on the intensity of alarm response on day 3 (Table 2). Effects of the immediate response of snails to the predator used on day 3 and the interaction term were not significant.

DISCUSSION

In Experiment 1, the alarm responses of apple snails to a predator were more intense when the snails had been exposed to both the predator and crushed conspecifics than the predator alone (Figs. 1, 2). The response was independent of the identity of the predator (carp versus turtle). These results indicate that predator avoidance is learned in a broad sense, which includes the possibility of associative learning and sensitization. This learning lasted at least 1 day (up to day 3), but the memory became weakened within 3 days (up to day 5).

Some freshwater pulmonate snails (Dalesman et al., 2006; Turner et al., 2006) and a marine caenogastropod (Rochette et al., 1998) are able to learn to avoid predators. Although there have been no reports regarding learned predator avoidance in freshwater caenogastropods, this study indicates that they are able to learn to avoid predators. Given this, learned predator avoidance may be important to snails in freshwater environments, and probably in marine environments as well.

The results of Experiment 2 indicate that the snails responded with greater intensity when conditioned to both a predator and the crushed conspecifics, then exposed to the same predator, as opposed to another species (“consistency of the predator” in Table 2). This suggests the use of associative learning with respect to a particular predator. We note only one other study with double predator conditioning, conducted in a minnow, *Pimephales promelas* (Chivers & Smith, 1994). However, we exposed conditioned prey individuals only once to one of the predators in the recognition trial unlike the previous study. Using this experimental design and two-way ANOVA, we could statistically distinguish the effect of associative learning (indicated as effect of “consistency of the predator”) from other components such as sensitization. One-way ANOVA followed by multiple comparisons is not the best test, because our hypothesis was the involvement of associative learning in general and not associative learning to each of the predator species.

In general, prey species have various innate and learned components of predator avoidance (Turner et al., 2006; Ferrari et al., 2007, 2008). Therefore, the occurrence of associative learning does not deny the possibility of sensitization or an innately higher response to one predator than another. During the conditioning trial in Experiment 2, when the snails experienced the predators for the first time, the response of

the snails to the carp was significantly greater than to the turtle. This result suggests that the innate response of the snails differed with respect to the identity of the predator, in the presence of crushed conspecifics. We cannot fully exclude the possibility that the odour from crushed conspecifics reached experimental snails more quickly in the carp treatment, if the carp moved more actively than the turtle. However, we think this unlikely, because the tank water was always mixed by the current caused by aeration.

In most reports on learned predator avoidance (e.g., Wisenden & Millard, 2001; Ferrari et al., 2005; Dalesman et al., 2006; Turner et al., 2006; Ferrari et al., 2006, 2007, 2008; Hazlett, 2007), test animals are exposed to the odour of the predator as a stimulus. We used live predators to simulate attack, as did Rochette et al. (1998). Thus, the snails could perceive not only the odours from the predators but also other cues such as their movement. Although this method does not allow conclusions regarding the cues used for predator recognition, it is more realistic of actual predation events in the field (Aizaki & Yusa, 2009). Our method also allows for the possibility that learning requires multiple stimuli, such as chemical and mechanical stimuli, acting together.

In summary, our study has shown that predator recognition in the apple snail has learned components. Among these, associative learning is the most parsimonious explanation. Further studies are required as various innate and learned components interact in a complicated manner to form predator recognition (Turner et al., 2006; Ferrari et al., 2007, 2008).

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